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Specific Reversible Concentration of Amino Acids in E. coli

by
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Introduction

In the present memoir, we are describing systems whose activity is accomplished by accumulation in the cells of Escherichia coli of certain exogenous amino acids. These systems seem to control the entrance into cellules of exogenous amino acids, and in consequence, their incorporation into proteins. On the contrary, these systems seem not to intervene in the metabolism of endogenous amino acids. By their kinetic properties and specificity, these systems belong to those which Rickenberg, Cohen and Monod describe elsewhere (15) and which control the penetration of B-galactosides in Escherichia coli. Putting these observations together conveys an experimental confirmation of the hypothesis often envisaged, that certain catalytic systems functionally specific, distinct from so-called metabolic enzymes, control penetration of certain substrates into the cells.

Certain results described in the present memoir have been published in the form of a preliminary note (14) and Britten, Roberts and French (23) have also recently described the accumulation of amino acids in E. coli. We shall see that the interpretation given by Britten et al, akin to that of our preliminary note, was entirely different from that which we propose now, and should be abandoned.

Experimental Part

Stock Used. -- Escherichia coli, stock K 12S, wild type. Valine-resistant mutant of K 12S, isolated by selection on valine (4). Mutants of E. coli exact valine (ML 328 f and M-28-62), leucine (ML 328 o), isoleucine (M 97-21) and methionine (ML 304 d).

Radioactive DL-valine.-- DL-valine 4-4'-^{14}C was furnished us by the Commissariat of Atomic Energy and had a specific activity of 1,33 millicurie per millimole. Under our conditions of counting, later defined, the radioactivity of valine corresponds to 10^5 impulses-minute/mole^{-6}, which corresponds to an efficiency count of 3,6 p. 100.

Non-Radioactive amino acids and other substances.-- Racemic and optically active amino acids of commercial origin were used. Valinamide, acids a-amino-B-butylheptanoique (dibutylalanine), a-amino-B-phenyl phenylpropricnique (B-B-diphenylalinine), a-amino-B-benzylphenylbutyrique (B-B-dibenzylalanine) and 6-methyltryptophane have been synthesized by Dr. J. Amatol, Dept. of Organic Chemistry of the Institut Pasteur. The acid a-amino-a-methylvalerian was given us by Dr. H.A. Krebs. Peptides were furnished by Drs. R. O. Roblin, J.K. Hinman, S.W. Fox, R.L.M. Synge and J. P. Greenstein.

Culture medium.-- Stock was transplanted every week on medium S3 (PO_4H_2K: 13,6g; $SO_4(NH_4)_2$: 2 g; $SO_4Mg, 7H_2O$: 0,2 g; $SO_4Fe, 7H_2O$: 0,0005 g; KOH q.s.p. pH 7,00; double distilled water q.s.p. 1000 ml.). To the medium was added succinate of K to 0,25 p. 100. In certain cases, we used stock not adapted to growth on succinate; glucose was then added to the medium to 0,2 p. 100. The same synthetic medium was used for the experiments.

Experimental Technique.-- The experiments are in a medium agitated with air. A culture of E. coli in exponential phase is diluted by medium 63 in the presence or absence of 5-methyltryptophane (5-MT) 5×10^{-4} M. Immediately after thermal equilibration (if 5-MT has not been used) or after thirty minutes (if in the presence of 5-MT), one adds to the culture the radioactive valine in known concentration and removes after a given time a sample of 5 ml which is drawn through a centrifuge tube previously chilled. Centrifuge at 0°C at 18,000 t.p.m. for five minutes; eliminate all liquid floating on the surface by aspiration with a Pasteur pipette, dry the tube with filter paper and dissolve the bacterial residue with 1,5 ml of distilled water. When it has been five minutes in a water bath, at 100°C, it is centrifuged and the surface liquid is decanted. Experience shows that by this method all radioactivity not incorporated in the proteins is extracted (that is, non-precipitable by trichloracetic acid). If one wishes to study the influence of another substance on the quantity of valine previously fixed, one adds this substance to the culture immediately after the first removal and takes another sample after a given time of contact; this sampling is treated below.

Spread 3 ml of surface liquid (corresponding to the bacteria contained in 1 ml of the suspension in the experiment) on small aluminum cups 15 mm in diameter and 3 mm in height, dry under an infrared lamp, and determine the radioactivity with the aid of a Geiger counter. The background noise varies between 12 and 14 impulses/minute. All samplings were double and the number of impulses counted for each sampling was always higher than 1000. In the conditions described, there was no auto-absorption and the impulses counted were proportionate to the total radioactivity of the sample. The reproductibility was better than 5 p. 100. Very radioactive samples were diluted so that the exposed radioactivity on the cup represents less than 3000 impulses/minute.

When one wishes to determine also the radioactivity incorporated in insoluble form, the residue centrifuged after boiling is rewashed and put again in suspension in 1,5 ml of distilled water, of which 0,3 ml are spread double. The measured radioactivity corresponds to 1 ml of the original culture. This represents the incorporation into proteins. In effect Roberts et al (3) have shown that exogenous valine contributes only to the valine and leucine of proteins and is not incorporated in non-protein fractions. We have verified that the radioactivity of the spread suspension is equal to that of the corresponding cold trichloracetic precipitate.

For an interpretation of the results, one must take into consideration the fact that a certain fraction of measured radiation (contamination) corresponds to the quantity of valine present in the liquid volume constituted by aqueous space (1) of bacteria, plus the volume of interstitial liquid and the volume of liquid adhering to the sides. We shall see by the following that the phenomenon of concentration which we are going to describe is suppressed by the 2-4-dinitrophenol. One can then, in the presence of this inhibitor, determine the liquid volume corresponding to this contamination. Under our experimental conditions, our results show that this contamination corresponds to a volume varying between 0,001 and 0,005 ml. In the case studied here, the contamination is negligible in relation to the pecific fixation just as the external concentration of DL-valine is inferior to 10^{-4}M. In our experiments, we have never passed this concentration and, as a consequence, we have not had to introduce a correction for the intracellular concentration.

The concentration of exogenous valine in the bacteria being, as we shall see, a reversible phenomenon, the quantity of intrabacterial valine concentrated is a function of concentration in the external medium, and experiments should be made in conditions where external concentration does

not vary noticeably. When it was not possible to operate in conditions where the proportion of fixed valine was negligible in relation to the total valine, the necessary correction was made for evaluation of actual external concentration.

We recall that the aqueous space, water (3), is defined as the volume of bacteria in which concentration of a metabolite is identical to its concentration in external medium.

The optical density of microbial suspensions is determined by means of a Mounier electrophotometer and the quantity of bacterial nitrogen per ml is calculated with the aid of a coefficient of conversion determined experimentally. Likewise, the number of bacteria by unit of volume is deducted from the optical density, also by means of a coefficient of conversion determined during the exponential phase and corresponding to a weight of 7×10^{-7} ug per average bacteria.

5-methyltryptophane has been used in a certain number of experiments so as to permit study of the kinetics of concentration in conditions where the total capacity of concentration per unit of volume remains constant in the course of growth of the bacterial mass. In effect, if one adds to the cultures of E. coli in exponential phase of growth, 5-MT in a concentration of 5×10^{-4}M, their optic density continues to increase (more slowly however than in normal cultures), but their capacity of valine concentration remains constant per unit of volume of culture, as we shall see in the course of the work.

Results

A. Specific reversible intracellular concentration of exogenous L-valine

Concentration of radioactive L-valine.-- Here is a typical experiment. Stock K 12S. Concentration of radioactive DL-valine: 5×10^{-6}M, or 250 impulses/minute ml corresponding to isomer L. After a minute of contact at 37°C, one finds 115 impulses-minute in bacteria of 1 ml of culture, which

represented a weight of 188 µg, or a humid weight of 1 mg and a volume of 10^{-3} ml, if one admits that bacteria have a density of 1. You have then realized a concentration of:

$$\frac{115}{250 \times 10^{-3}} = 460 \text{ times}$$

At the concentration of valine used, the contamination is about 0.6-3 impulses-minute.

Time necessary for maximum concentration of L-valine. -- If the experiment is done in the presence of 5-MT, which inhibits the growth of the total capacity of concentration in the bacterial mass in growth, concentration is maximum after a minute of contact if the experiment is done at 37°C. If the experiment is done at 0°C, about twenty minutes is necessary to obtain the maximum concentration. However, the absolute value of the maximum concentration at the two temperatures is identical (table I). In the following, the time of contact chosen has been for one minute at 37°C.

Proportionality between quantity of bacteria and quantity of concentrated radioactive L-valine.-- If the experiment is done with a sufficient quantity of valine (5×10^{-5} M of DL-valine), the concentrated radioactivity is strictly proportional to the quantity of bacteria used, in a zone ranging from 3 to 30 µg N bacteria/ml (fig.1). We shall call specific capacity of concentration the capacity of concentration by unit of bacterial weight, in contrast to the total capacity of concentration, defined as the capacity of concentration of the unit of volume of culture.

Balance of the reaction. Affinity of valine for bacteria. -- By determining the quantity of valine concentrated as a function of the quantity of external valine, one obtains a curve of typical adsorption. The capacity of maximal concentration of the system corresponds to

$3,6 \times 10^{6}$ molecules of L-valine per average bacteria (eleven independent determinations, the extreme values being $2,2 \times 1\ 10^{6}$ and 5×10^{6} molecules per bacteria). It is reached at the beginning of an external concentration of $5 \times 10^{--5}$ M of L-valine. (fig. 2)

The constant of dissociation apparent in the system is very weak, by order of KL-valine = $3 \times 10^{--6}$M (fig. ?). This value has been determined in correcting the values of external concentrations (table II). In effect, for the weak concentrations of valine, the phenomenon of intracellular concentration bows perceptibly to the external concentration. The specific capacity of concentration of the system and the constant of dissociation are identical for the wild type of K 12 S and for the valine-resistant mutant.

We see that our results are expressed in L-valine. We shall see later, in effect, that only L-valine is concentrated.

Chromatographic analysis of the extracted material by boiling of bacteria having concentration of valine for one minute shows that this material consists only of valine, unaltered.

Conditions necessary for concentration.-- Specific concentration of L-valine requires a source of energy.

In table III, we see that the experiment is made in the absence of an external source of energy (succinate), the specific concentration is reduced by 71 p. 100. If the experiment is done in the presence of nitrite of sodium $1,5 \times 10^{--2}$M or of 2-4 dinitrophenol 10^{-3}M, the specific concentration is reduced respectively by 84 and 92 p. 100. If one adds nitride to a culture already having a concentrate of radioactive valine, one observes a slow loss of valine previously concentrated: after thirty minutes, the bacteria still contain 44 p. 100 of the valine which they had concentrated before the addition of nitride.

Specificity of fixation of L-valine. Competitive deplacement.

Hirsch and Cohen (5,6) have shown that L-leucine and L-isoleucine, analogous structurally to valine, are antagonists of this amino acid in Escherichia coli.

If one adds to bacteria having already a concentrate of radioactive valine some leucine or isoleucine in sufficient quantity, the valine is almost entirely expelled from bacteria in one minute at 37°C. It is the same case with norleucine (table IV).

A quantitative study of this phenomenon shows that the relation antagonist / valine necessary to obtain a deplacement of 50 p. 100 of radioactive valine is constant, which indicates a competitive reaction. In consequence, it occurs as if the valine were concentrated thanks to a system by which leucine, isoleucine and norleucine would have equal affinity (table V). This system is specific; in fact, amino acids without evident structural relation with valine, such as phenylalanine, proline, threonine or methionine do not drive out the valine previously concentrated, or eliminate only a weak fraction and only if they are used in high concentration (table IV). We shall later discuss the question of knowing if the phenomenon of concentration is due to stoichemistry or to a system of enzyme properties not intervening as catalysor. For the moment we shall study the properties of specificity of these "acceptors" without prejudging their nature or their precise function in the system of concentration.

Isoleucine and leucine are the most effective antagonists (50 p. 100 of inhibition for a ratio antagonist/valine = 0,5--1,0) since norleucine is much less active (50 p. 100 of inhibition for a ratio norleucine/valine $>$ 10). The radioactive L-valine also deplaces the radioactive valine previously concentrated.

The order of addition is absolutely indifferent; isoleucine, for example, exercises the same effect of competition whether it has been added before or after the valine. This fact indicates that the specific intracellular concentration is a reversible process (this does not prejudice the reversibility or not of different reactions which may constitute it), which is already evident from the fact that one can attain a measurable balance.

Only the composites of series L have affinity for specific acceptors: in fact, D-valine, D-leucine and D-isoleucine do not expel the radioactive valine (table VI); from this it is deduced that in our experiments, done with DL-valine, only the isomere L is concentrated, which justifies that our results are expressed with no regard for L-valine.

The substitution of the amino group or carboxyl group removes the affinity for acceptors: DL-N-monomethylvaline and DL-valinamide do not deplace the valine previously concentrated. If the two methyl residues of the isopropyle group of valine are replaced by the residues of butyl, phenyl or benzyl, the affinity also disappears. Likewise, the acid a-amino-a-methylvalerian is deprived of affinity (table VI).

In summary, only the composites of the L series having a carboxyl group and an amino group not substituted, and a not indifferent configuration of their extreme non-polarity, present affinity for the system of specific concentration of the L-valine.

Peptides containing valine, leucine and isoleucine have practically no affinity for the specific acceptors of L-valine, which corresponds to what has already been seen with valinamide. The most active of those which have been tried, glycyl-L-isoleucine, provokes only an inhibition of 50 p. 100 for a ratio peptide/L-valine = 100, since this same inhibition in the case of L-isoleucine is obtained for a ratio L-isoleucine/L-valine= 0,5 — 1,0 (table VII).

The weak inhibition by the peptides does not seem elsewhere to have a competitive character. Elsewhere the effect is due in part to rapid hydrolysis of peptides by the cultures of E. coli, which is not negligible, even in a minute. Peptides not hydrolysed (D-valyl-L-valine, L-valyl-D-valine, DL-valinamide) are absolutely without effect (table VII).

Independence between specific intracellular concentration of valine and synthesis of proteins.

It is not necessary to have synthesis for specific concentration to take place, as is shown by the following facts:

Specific concentration of the valine is not affected by the absence of other amino acids: for example, it takes place in a mutant requiring methionine as well in the absence as in the presence of methionine; it is not affected by the presence of analogous structures, such as 4- and 5-methyltryptophane (table VIII), which inhibit synthesis of tryptophane in the beginning of the indole and of serine (7,8) or B-2-thienylalanine which inhibits utilization of phenylalanine (9). Finally, in the presence of chloramphenicol, incorporation in the proteins is totally suppressed, but the specific intracellular concentration is not affected (table IX).

The specific concentration is produced much more rapidly than incorporation of valine in the protein bacteria. In fact, the concentration attains its maximum in about a minute, since the incorporation is still negligible.

Effect of weak antagonistic concentrations.

Among the sub-inhibitive concentrations of L-leucine and L-isoleucine, one observes an increase, in accord with the demonstration without inhibitor, in the quantity of L-valine reversibly concentrated; this increase becomes less important in proportion as the antagonistic concentration is increased (table V). This remarkable phenomenon, for which we can furnish no explanation, finds an exact parallel in the effect of concentrations of weak antagonists on

the growth of mutants requiring valine, as we shall see in the second part of this work.

The aggregate of observations discussed in Table X shows that the cells of E. coli reversibly concentrate exogeneous valine, that this phenomenon of concentration causes a specific system and that it is coupled with the energetic metabolism. We have been able to show the existence of an analogous phenomenon, and in consequence of systems of specific concentration for methionine (^{35}S), for tyrosine ($2\text{--}^{14}C$). and for phenylalanine ($3\text{—}^{14}C$) (Table X and XI).

In the second part of this work we are going to study the correlation between specific reversible intracellular concentration and utilization, or the effects of exogeneous valine, leucine and isoleucine on different strains of E. coli.

B. -- Interpretation of the effects of exogeneous valine, leucine and isoleucine on the growth of E. coli K 12 S and on the growth of mutants of E. coli requiring amino acids.

Case of wild strain E. coli K 12 S.

We know that the growth of wild strain of E. coli K 12 S is inhibited by L-valine and re-established by L-isoleucine (10). Umbarger (11) thinks that the extreme sensibility of this organism to L-valine (a concentration of 5×10^{-7}M inhibits growth at the beginning of inoculum of 10^6bacteria) is due to the fact that synthesis of isoleucine in this organism would be relatively slow. He supposes that the limiting reaction in this synthesis would be very early, previous in any case to the stage of homoserine: in fact, homoserine, threonine and the other composites implicated as precursors of isoleucine, and isoleucine itself, increase notably the rate of growth of K 12. Cohen and Hirsch (notes not published) have shown effectively that the activity of the synthesiting homoserine was less high in K 12 than in other strains of E. coli.

However, the growth of K 12 S, inhibited by valine, can also be re-established by L-leucine (instead of L-isoleucine) in a competitive manner (12), as we have already verified.

The parallel between these antagonism and those which reveal specific reversible fixation is remarkable: the rapport isoleucine/valine, necessary to expel 50 p. 100 of valine and to re-establish growth of 50 p. 100, is sensibly the same. There is no doubt that the toxic effect of valine on growth of K 12 is not conditioned by its specific concentration. It is equally evident that the effect of isoleucine, restoring growth, is due to its antagonism face-to-face with the phenomenon of concentration. Nevertheless, it must be supposed that the inhibitive effect of valine, if it is conditioned by concentration, is not, however, an inherent consequence, since the valine-resistant mutant of K 12 presents the phenomenon of concentration, in conditions identical to the wild valine-sensible form. In conclusion, the toxic effect of valine is not necessarily linked to its antagonism with isoleucine.

Case of auxotrophic mutants requiring L-valine, L-leucine and L-isoleucine

Here again, antagonisms revealed by growth find a parallel with the phenomenon of concentration.

Hirsch and Cohen (6) have described competitive inhibition of growth of the strain ML 328c requiring L-leucine by L-valine and L-isoleucine. We have extended these observations to mutants requiring respectively L-isoleucine (M 97-21) and L-valine (ML 328l and M 48-62) for growth.

The growth of mutant M. 97-21 is competitively inhibited by L-valine although the relation L-valine/L-isoleucine necessary to inhibit growth is much higher than in K 12 (table XII).

The growth of mutants ML 328f and M 48-62 is competitively inhibited by L-leucine and L-isoleucine (5) and by DL-norleucine.

The growth of the valine resistant mutant of K 12S, which we have isolated by selection in presence of valine (4) is not affected by valine or isoleucine. Figure 3 shows that the rate of growth is identical in presence of these amino acids or in their absence.

According to Roberts et al (3), if one cultivates E. coli in presence of radioactive valine, 95 p. 100 of the valine incorporated in the proteins are radioactive.

With the valine-resistant mutant, we have followed the growth of the radioactivity of the protein as well as the increase of extractible radioactivity as a part of the increase of the bacterial mass, in a culture made in presence of radioactive DL-valine 10^{-4}. One sees (figure 4) that in these conditions, the total radioactivity incorporated increases linearly in accordance with the growth of the bacterial mass. The slope to the right measures the specific radioactivity of the valine in the synthetized proteins from the moment of the addition.

When the cultures are made in presence of growing concentrations of isoleucine, the specific radioactivity of the valine in the proteins diminishes until it is almost annulled. (figure 4). As the presence or absence of valine or isoleucine is without effect on the growth of these bacteria (figure 3), it is not probable that their tenure in valine varies notably; the valine incorporated in presence of isoleucine is then of the non-radioactive valine synthesized by the bacteria at the beginning of the source of carbon employed (succinate).

In the case of the valine-resistant strain, isoleucine still replaces the valine, but there results no advantage or disadvantage to the culture, the bacteria being neither sensible to valine as K 12S wild, nor requiring valine as L 328f. We arrive then at a single explanation of the valine-isoleucine antagonism in K 12, in mutants requiring E. coli and in strains

which are neither sensible nor exacting. The common phenomenon is competitive blockage of the system of specifi concentration responsible for concentration. Table XIV gives a resume of the situation.

Effect of Weak Antagonistic Concentrations on Exacting Mutants.

With numerous mutants, if non-optimal concentrations of the amino acid factor of growth are used, it is frequently observed that the addition of certain other amino acids, in particular those having a structural relation with the factor of growth, are translated by a final yield higher in bacteria (sparing effect).

In the particular case which interests us, one notes an effect of sparing of isoleucine and leucine on the growth of mutant ML 328f which exacts L-valine: for a concentration of L-valine 5×10^{-6} M. and a concentration of isoleucine x, 5×10^{-6} M, one obtains, after 18 hours of growth, a final yield in bacteria higher than that obtained with a concentration of 10^{-5} M. of valine along (table XV). Compare this result with the effect of weak concentrations of L-isoleucine which increase the quantity of L-valine concentrated specifically and reversibly (table V). We cannot, for the time being, provide any interpretation of this phenomenon. But one sees here again, the parallelism between the effects of isoleucine in growth and its effects observed on the system of specific concentration is entire and continued in certain peculiarly characteristic details.

Attempted Explanation of Behavior of Peptides in Mutants of E. coli.

Hirsch and Cohen (6) have stated that if one grew strain ML 328c, exacting leucine, in presence of peptides containing leucine (glycyl-L-leucine or L-leucyl-glycine), the growth became insensible to L-valine and to L-isoleucine. These results have been extended to ML 328f, exacting L-valine. In this organism the growth is competitively inhibited by L-leucine or L-isoleucine. By contrast, the growth of peptides containing L-valine is

is not affected by these inhibitors (table XVI).

We have for the present proposed as possible interpretation that the inhibitors prevent peptide synthesis, but that if a peptide were furnished preformed, the other peptides to a valine could be snythesized by transpeptidation. The results which are discussed above show that inhibition of growth of exacting mutants is due to a competition for the system of concentration. The peptides escape this competition, which conforms to results indicating that they themselves have no affinity for the system in question (table VII). But this leaves unanswered the question of knowing by what way they are engaged in the metabolism of protein synthesis.

Discussion

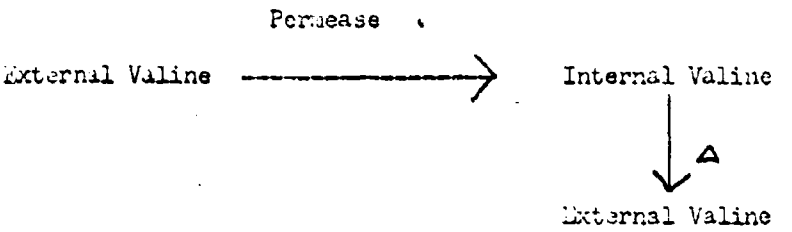
We have just shown that the cellules of E. coli are capable of actively accumulating external valine, methionine, tyrosine and phenylalanine. It is entirely possible to think that this phenomenon is produced by all natural amino acids. As balance, the internal concentration can attain several hundred times the external concentration. Our results show that this phenomenon of intracellular concentration is reversible (experiments in balance and deplacement), that it is linked to the energetic metabolism (experiments at 0°C and at 37°C, inhibitions by nitrate and by dinitrophenol); that it is highly specific (competitive deplacement by analogous composites only) and that there must exist some mechanism or system of distinct concentration for each amino acid or type of amino acid.

The kinetic and specific properties of the system of concentration of valine lead to envisaging two types of different models to take account of the phenomena.

a) Stoichiometrical scheme. -- The valine would be reversibly fixed in stoichiometrical proportion on specific acceptors for which isoleucine would have equal affinity, thus

Valine / S = Valine — S

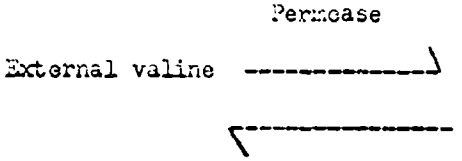
b) Catalytic scheme. — In this hypothesis, a specific system would catalyse the passage of external valine across the osmot cellular barrier. Accumulated valine would be liberated by a process △ which would not interrupt the system in question and whose velocity would be proportional to the quantity of internal valine.



In these conditions, the internal concentration of valine to the balance, is proportional to the activity of valine-permease. The addition of isoleucine would inhibit competitively the permease and valine already concentrated (and not fixed) would be transformed into external valine (which is to say expulsed) under the action of △ .

The two schemes proposed explain all the phenomena of saturation and competitive deplacement. None of the kinetic results permit choosing one hypothesis over the other. From other considerations we are led to prefer the catalytic system, but before accepting them, let us show that other models, a priori similar, cannot be retained.

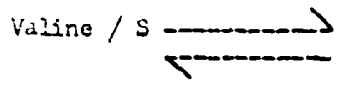
c) A first possibility would be a catalytic mechanism or the reversibility would be assured by the permease:



It is evident that such a scheme takes no account of either the competitive deplacement (the addition of isoleucine should stabilize the system at the state of balance attained at the moment of this addition) or of saturation.

If one completes this model thus

d)



permease being specific and S an acceptor non specific, saturation is explained, but not the competitive specific deplacement. If S is specific, one returns to the stoichiometrical scheme complicated by a permease.

In summary, we find ourselves in the presence of two acceptable models, the one stoichiometrical (a), the other catalytic (b).

In the system of reversible intracellular concentration of B-D-galactosides and in that which we are discussing at present, we have first of all considered a scheme of the stoichiometrical type. (13,14). In later experiments (15,16) we have been led to the conclusion that, in the case of the system of B-galactosides, the stoichiometrical scheme should be rejected. The very rigid analogy between properties of the system of concentration of B-galactosides and systems of amino acid concentration suggest looking for the interpretation of the latter preferably by the catalytic scheme. In order to consider only results concerning amino acids, it is necessary also that the admitted scheme explain in simple fashion the interactions between amino acids in different strains of E. coli. The catalytic model permits giving an explanation of these in eractions which is satisfying except in certain particularly characteristic details: in particular, it takes account, pursuant to a remarkable parallelism, of all phenomena of competitive inhibition observed in E. coli under the influence of valine, leucine and exogeneous isoleucine. The stoichiometrical model, on the contrary cannot explain these phenomena

except with the aid of arbitrary supplementary hypotheses; in particular, it does not explain how, in E. coli ML, wild type, exogenous isoleucine which does not affect the rate of growth and metabolism, controls the quantity of exogeneous valine incorporated in the proteins, without admitting that synthesized endogenous valine at the beginning of succinate does not pass through the specific sites.

This scheme is considered as preliminary. The general effect of the properties of the system of concentration, in particular the nature of the reactions of permease and $\triangle$ is not cleared, and it is voluntarily that we refrain from representing the catalytic scheme (b) in a more precise fashion. Particularly, in the simple scheme which we have proposed, we have not attempted to take account of the mechanism of energetic coupling, on a level with which inhibition by dinitrophenol and nitrate doubtless takes place. Now, one will have noted that the addition of nitrate to bacteria having already concentrated valine is shown by a very slow loss, since deplacement by an analog is extremely rapid. It seems then that metabolic inhibitors interfere not only with the reaction of entrance, but equally with the reaction of exit $\triangle$.

It would appear very likely that the specific catalytic systems of which we have demonstrated the existence are constituted essentially of specific proteins, perhaps proteins of the cellular membrane. Only a reasonable hypothesis is concerned, but we recall that in the case of galactoside-permease, this system is inductible in conditions identical to those which permit induction of B-galactosides itself and that the formation of the system is inhibited by chloromycetin. We have obtained analogous results, so far as formation of methionine-permease is concerned, which for reasons which we will explain elsewhere, lend themselves to this type of experiment. There is thus no doubt that the activity of galactoside-permease and, by extension, of amino acid-permeases, is not connected with the presence of specific proteins.

Works now classics, of Gale (1) and those of Halvorson and Spiegelman (2) have shown that there is an accumulation of certain amino acids in staphylococci and in yeasts, and that this accumulation is linked with utilization of metabolic energy. In these cases, however, intervention of specific catalytic systems has not been sought.

It is known that very frequently phenomena of selective permeability have been supposed to explain certain contradictions or difficulties in the interpretation of activity of metabolic substrates. Barrett, Larson and Kallio (17), Kogut and Podovski (18), Green and Davis (19) have analyzed such a phenomena in the case of citrate in Pseudomonas fluorescens and aerobacter aerogenes. It is a question there of induced biosynthesis of a system permitting accession of citrate to enzymes of the Krebs cycle. However, in this case, there seems to be no accumulation of intracellular citrate and it is not possible to show as direct evidence the reaction of transfer. Let us note in conclusion, that Britten, Roberts and French (23) using techniques very different from ours, have independently given evidence of the phenomenon of specific concentration of amino acids in E. coli. They propose an interpretation of the stoichiometrical type, akin to that of our earlier communication (14), an interpretation which we have now abandoned.

Very recently, Mathieson and Catcheside (22) have described, in Neurospora crassa, a non-reversible concentration of histidine which seems to be inhibited by a whole series of amino acids. This system could be analogous to that of E. coli, but with much less restricted specificity.

The transfer of different amino acids of series L (but not of series D) across rat intestine, has been described by Agar, Mira and Sidhu (21). This transfer is inhibited by 2-4 dinitrophenol and cyanide. It is a question of a phenomenon not directly comparable to that which we have studied since the

transfer in question takes place in extra-cellular space toward another extra-cellular space, across a tissue. But it is interesting to note that by reason of its reversibility, a mechanism such as we have described in E. coli could take equal account of a transfer across a mono-pluricellular layer. It could also take account of a transfer in connection with a drop in temperature of concentration or activity, with the only condition that the permease unequally distributed on the two surfaces of the cellular layer.

The advantage of a study of systems of intracellular concentration existing in E. coli for amino acids (14) and this work) and for B-D-galactosides (13, 15, 16)(and really for all glucides) resides in their reversibility and in their specificity. Their nearly simultaneous discovery suggested that systems of this kind are of great generality and an important capital in the physiology of microorganisms, if not of other cellules.

We have to thank M. Jacques Monod with whom we have had numerous fruitful discussions and Mlle. Marcelle Lannes for technical assistance.

Resume

There exists in Escherichia coli a series of specific systems responsible for a reversible concentration of exogenous amino acids, which precedes their incorporation into proteins. The properties of these systems explain a number of growth inhibitions and interactions between amino acids.

Table I - Time necessary for maximum concentration of radioactive valine

Température	Temps d'incubation (min.)	$ bactérien µg/ml cult.	Concentration optimale réversible/bactérien de 1 ml de culture	
			Impulsions-mn	moles x 10^{-11} L-valine
1 - 37°C	1	24,5	358	179
	30	27,7	391	195
	120	36,7	355	177
2 - 0°C	1	24,5	64	32
	30	24,5	228	114
	120	24,5	366	183
	180	24,5	372	186

E. coli K 12, mutant valine-resistant. Experiments at 0° and at 37°C are made in the presence of 5-AT 5.10^{-4} (the valine is added after a contact of thirty minutes of culture with 5-AT). DL-valine radioactive: 10-4..

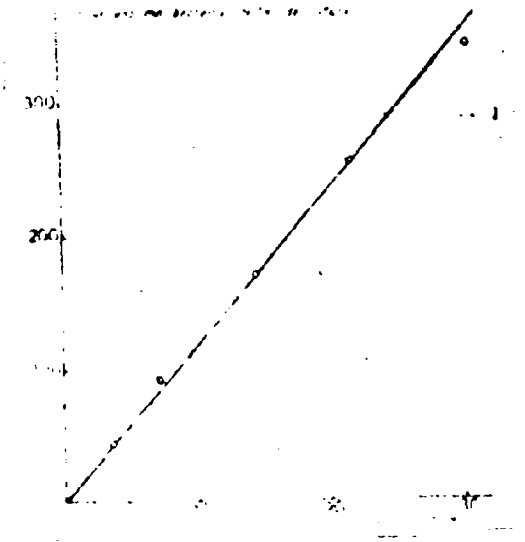


Fig. 1. — Proportionality between the quantity of bacteria and the quantity of concentrated radioactive L-valine. E. coli K 12 S.

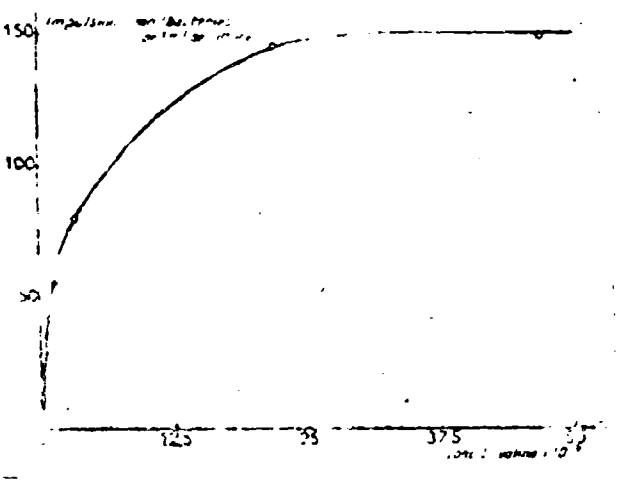


Fig. 2. — Effect of external concentration of valine on specific reversible concentration. E coli K 12 S.

Table II. -- Affinity of L-valine for the system of specific concentration (E. coli K 12 S).

Concentration externe de L-valine M	Impulsions-sec/ml correspondantes	Impulsions-sec fixées /ml de culture	Impulsions-sec de L-valine externe corrigées	Concentration externe de L-valine corrigée
$2,25.10^{-6}$	62	20	42	$8,5.10^{-7}$
$2,5.10^{-6}$	125	56	69	$1,4.10^{-6}$
5.10^{-6}	250	80	170	$3,4.10^{-6}$
$2,5.10^{-5}$	1.250	145	1.105	$2,2.10^{-5}$
9.10^{-5}	8.500	360	8.500	$8,7.10^{-5}$

Table III. -- Effect of the absence of a source of energy and of the addition of inhibiting agents of phosphorylations on the specific concentration of L-valine.

Expérience	Conditions expérimentales	Bactéries/ml	Concentration spécifique réversible/bactéries de 1 ml de culture		Inhibition p. 100
			Impulsions-mn	moles x 10^{-11} L-valine	
I	DL-valine 5.10^{-5} M				
	avec succinate	18,9	375	163	
	sans succinate	14,2	92	40	71
II	Milieu complet				
	a) - DL-valine 5.10^{-5} M	15,1	234	107	
	b) - N_3Na $1,5.10^{-2}$ M contact 5 mn, puis DL-valine 5.10^{-5} M	14,0	32	16	84
	c) - comme b, mais avec 2,4 dinitrophénol 10^{-3} M	14,4	16	8	92
III	Milieu complet				
	DL-valine 10^{-4} M	15,5			
	contact 1 mn, puis	15,5	276	112	
	N_3Na $1,5.10^{-2}$ M				
	contact 1 minute		149	70	
	" 5 minutes		139	60	
	" 30 minutes	15,9	99	50	44

E. coli K 12 S. Concentration of radioactive DL-valine indicated for each experiment. In experiment 1, the bacteria are centrifuged, washed twice with a medium without succinate and divided into 2 flasks, one containing succinate to 0,25 p. 100, the other containing no source of carbon. The two cultures are agitated for thirty minutes (to extract the reserves of bacteria in the medium without succinate) having the addition of valine. Experiments I and III were made in the presence of 5-MT5.10^{-4}M, experiment II without 5-MT.

Table IV. -- Deplacement of valine previously concentrated by E.coli K 12 S by other amino acids.

Fiole	Additions	Concentration dans les bactéries de 1 ml de culture		Inhibition p. 100
		Impulsions-mn	moles x 10^{-12} L-valine	
A	0	329	165	
	L-isoleucine 5.10^{-5}M	54	27	83
B	0	293	147	
	L-isoleucine 10^{-5}M	38	19	87
C	0	309	155	
	L-leucine 5.10^{-5}M	43	22	86
D	0	305	153	
	L-leucine 10^{-5}M	35	17	89
E	0	291	146	
	L-valine non radioactive 5.10^{-5}M	99	50	66
F	0	304	152	
	L-valine non radioactive 10^{-5}M	41	21	86
G	0	307	154	
	DL-norleucine 10^{-5}M	67	34	80
H	0	300	150	
	L-phénylalanine 10^{-5}M	288	144	4
I	0	325	163	
	L-proline 10^{-5}M	377	189	0
J	0	349	173	
	DL-méthionine 10^{-5}M	315	158	9
K	0	341	171	
	DL-méthionine 5×10^{-5}M	319	160	7
L	0	347	174	
	DL-méthionine 10^{-4}M	293	147	16
M	0	305	153	
	DL-méthionine 10^{-3}M	206	103	32
N	0	300	150	
	DL-thréonine 10^{-3}M	150	75	50

N. bacteria/ml of culture: 27 µg. Concentration of radioactive DL-valine: 10^{-5}M = 5.10^{-6}M in L.

Table V. — Competitive character of deplacement of radioactive L-valine by L-isoleucine and by DL-norleucine

Exp.	Concentration L-valine radioactive M	Concentration L-isoleucine M	Concentration DL-norleucine M	Concentration par les bactéries de 1 ml de culture		Rapport L-isoleucine/L-valine pour une inhibition de 50 p.100
				imp./mn	moles x 10^{-12} L-valine	
I	5.10^{-6}	0	-	241	121	
		5.10^{-7}	-	340	170	
		10^{-6}	-	314	157	
		$2,5.10^{-6}$	-	98	49	sa. 0,5
		5.10^{-5}	-	40	20	
	5.10^{-5}	0	-	430	215	
		5.10^{-7}	-	568	284	
		10^{-6}	-	511	256	
		5.10^{-6}	-	473	237	
		10^{-5}	-	384	192	
		5.10^{-5}	-	240	120	sa. 1,0
		10^{-4}	-	105	53	
		5.10^{-4}	-	70	35	
II	5.10^{-6}	-	0	189	95	
		-	10^{-4}	86	43	sa. 10
		-	5.10^{-4}	61	31	
		-	10^{-3}	54	27	
	5.10^{-5}	-	0	395	198	
		-	10^{-4}	389	195	
		-	5.10^{-4}	292	146	
		-	10^{-3}	180	90	sa. 10

E.coli K 12 S.N. bacteria/ml of culture = 18 ug (exp.I) and 16 ug (exp II)

Table VI. — Structural conditions requisite for the competitive deplacement

Additions à	Concentration dans les matrices de 1 ml de culture	
	Impulsions/minute	moles x 10^{-11} L-valine
0	297 (*)	149
L-isoleucine 5.10^{-5}	54	27
L-leucine 5.10^{-5}	41	21
L-valine non radioactive 10^{-3}	40	20
D-valine 10^{-3}	374	187
D-isoleucine 10^{-3}	325	163
D-leucine 10^{-3}	334	167
DL-dibutylalanine 5.10^{-5}	300	150
DL-dibutylalanine 10^{-3}	237	119
DL-N-acetylvaline 10^{-3}	356	178
DL-valinamide 10^{-3}	302	151
Acide DL-a-amino-a-méthyl valérianique 10^{-3}	270	135
DL-diphénylalanine 10^{-3}	369	184
DL-dibenzylalanine 10^{-3}	350	175

E. coli : 12 S-A bacteria: 27 ug/ml; DL-valine radioactive: 5.10^{-6} M. in L.
*This value is a median value; results without addition of each experiment differ only to a maximum of ± 7 p. 100.

Table VII. -- Effect of peptides on radioactive valine previously concentrated.

Additions M	Concentration dans les bactéries de 1 ml de culture	
	Impulsions/minute	moles x 10^{-11} L-valine
0	188	94
L-valine non radioactive 10^{-3}	25	13
L-leucine 10^{-3}	25	13
L-isoleucine 10^{-3}	29	15
DL-valyl-DL-alanine 10^{-3} ($2,5.10^{-4}$ en L-L)	127	64
L-valyl-D-valine	188	94
D-valyl-L-valine	185	92
L-lysyl-L-valyl-L-phényl-alanylglycine 10^{-3}	190	95
L-prolyl-L-leucine	142	88
Glycyl-L-isoleucine 10^{-3}	190	95

N bacteria: 18 ug/ml; DL-valine radioactive: 5.10^{-6} in L.

Table VIII. — Absence of effect of 5-methyltryptophane on the specific reversible concentration of valine.

Fioles	DL-valine radioactive M	Concentration spécifique réversible/bactéries de 1 ml de culture			
		Série A		Série B	
		Imp./mn	moles x 10^{-11} L-valine	Imp./mn	moles x 10^{-11} L-valine
1	$1,25.10^{-6}$	20	10	19	10
2	$2,5.10^{-6}$	57	29	56	28
3	5.10^{-6}	80	40	86	43
4	$2,5.10^{-5}$	145	73	153	77
5	5.10^{-5}	149	75	154	77

E.coli K 12S. The vials of series A contain 5-MT 5.10^{-4} M (preincubation of 30 minutes*. Vials of series B contain none. M. bacteria/ml : 18 ug.

Table IX. — Absence of effect of chloramphenicol on specific concentration of radioactive valine

Temps minutes	Flacon sans chloramphénicol		Flacon avec chloramphénicol	
	Concentration spécifique réversible/bactérie de 1 ml de culture	Incorporation dans les protéines des bactéries de 1 ml de culture	Concentration spécifique réversible/bactérie de 1 ml de culture	Incorporation dans les protéines des bactéries de 1 ml de culture
	moles x 10^{-11} L-valine		moles x 10^{-11} L-valine	
1	145	75	140	3
30	-	988	-	7
90	-	2.088	-	10

E. coli K 12 S. Chloramphenicol is utilized to a concentration of 40 ug/ml and is left five minutes in contact with the culture before addition of valine. The specific reversible concentration is measured as usual, after a minute at 37°C. The two cultures are left in presence of radioactive valine to verify the absence of protein synthesis of chloramphenicol. Experiment without 5-HT.
Initial bacteria: 18 ug N/ml.

Table X. -- Specificity of deplacement of L-phenylalanine previously concentrated.

Flacle	Additions	Concentration dans les bactéries de 1 ml de culture	
		Impulsions/mn	moles x 10^{-11} L-phénylalanine
A	0	133	54
	L-phénylalanine 10^{-4}M	47	19
B	0	130	53
	D-phénylalanine 10^{-3}M	160	65
C	0	136	55
	L-isoleucine 10^{-2}M	118	48
D	0	132	54
	DL-β-phénylsérine	119	48
E	0	131	54
	Phénylpyruvate de Na 10^{-3}M	161	65
F	0	130	53
	DL-β-phényllactate de Na 10^{-3}M	117	48
G	0	136	55
	DL-parafluorophénylalanine 10^{-3}M	66	27

E.coli, mutant valine resistant of K 12. K bacteria: 19,8 ug/ml DL-phenylalanine radioactive 5.10^{-3}M. Specific activity: 122.700 impulses-minute/ mole^{-6}. Same experimental conditions as for valine. Time of contact of phenylalanine and inhibitor: one minute.

Table XI. Specificity of deplacement of L-Methionine previously concentrated.

Fioles	Additions	Concentration dans les bactéries de 1 ml de culture	
		Impulsions-minute	moles x 10^{-11} L-méthionine
A	0	302	77
	L-méthionine 10^{-4}M	77	20
B	0	307	77
	D-méthionine 10^{-4}M	307	77
C	0	299	76
	L-norleucine 10^{-4}M	115	29
D	0	299	76
	D-norleucine 10^{-4}M	301	76
E	0	300	76
	L-proline 10^{-3}M	240	61
F	0	304	77
	L-phénylalanine 10^{-3}M	253	65

E. coli K 12S. N. bacteria: 27 ug/ml. DL-methionine radioactive 5.10^{-5}M. Specific activity: 390.000 imp./mn/mole^{-1}. Same experimental conditions as for valine and phenylalanine.

Table XII. — Competitive inhibition by L-valine of growth of mutant of E. coli K 97-21, requiring L-isoleucine

L-isoleucine M	L-valine M	Croissance (unités Klett)	Index d'inhibition (*)
5.10^{-6}	0	8	
"	5.10^{-5}	10	
"	10^{-4}	4	
"	5.10^{-4}	0	
10^{-5}	0	40	
"	10^{-4}	32	
"	5.10^{-4}	24	
"	10^{-3}	9	100
5.10^{-5}	0	87	
"	10^{-3}	75	
"	5.10^{-3}	2	100
10^{-4}	0	99	
"	10^{-3}	77	
"	5.10^{-3}	66	
"	10^{-2}	15	100

(*) Defined as the relative inhibitor/factor of growth totalling suppressing growth.
For the conditions of this experiment and of those described in Table XIII, XV and XVI, see Hirsch and Cohen (6)

Table XIII. — Competitive inhibition of growth of mutant ML 328f requiring L-valine for its growth

L-valine M	L-norleucine M	Croissance (unités Klett)
0	0	0
10^{-5}	-	117
10^{-5}	10^{-6}	116
10^{-5}	10^{-5}	24
10^{-5}	5.10^{-5}	0
5.10^{-5}	-	109
5.10^{-5}	10^{-6}	105
5.10^{-5}	10^{-5}	109
5.10^{-5}	5.10^{-5}	97
5.10^{-5}	10^{-4}	2
10^{-4}	-	110
10^{-4}	10^{-5}	109
10^{-4}	5.10^{-5}	113
10^{-4}	10^{-4}	15
10^{-4}	5.10^{-4}	0

DL-norleucine has been used, but the isomere L alone being active, the concentration of this aminoacid is expressed in isomere L.

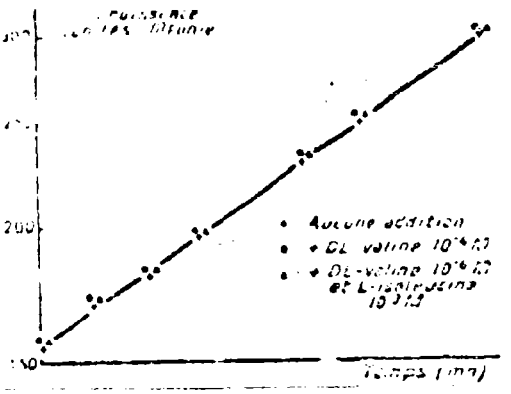


Fig. 3. — Growth of valine-resistant mutant of E. coli K 12, in absence of all addition, in presence of valine, and in simultaneous presence of valine and isoleucine. The same result is obtained with E. coli K, wild type.

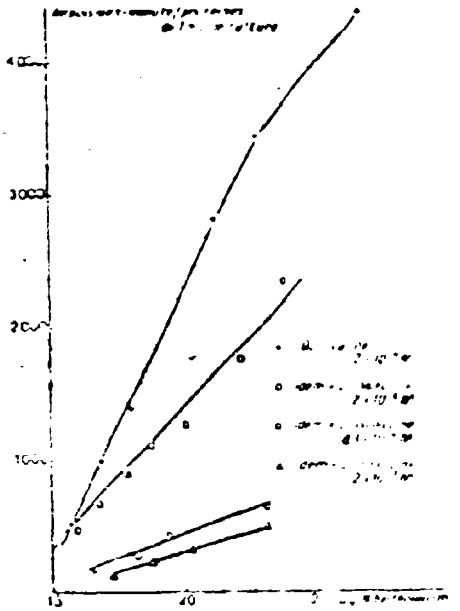


Fig. 4. — Incorporation radioactive of L-valine in absence and in presence of growing concentrations of L-isoleucine. Mutant valine-resistant of E. coli K 12. The same result is obtained with E. coli K, wild type.

Table XIV. — Resume of interactions between valine, leucine and isoleucine

Organisme	Nature	Antagoniste	Action de l'antagoniste	Effet sur la croissance
E 12 S	Valine-sensible	Isoleucine Leucine	Déplacement compétitif	croissance rétablie
ML 328 f	Valine exigeant	Isoleucine Leucine	Déplacement compétitif	croissance inhibée
B 77-V1	Isoleucine exigeant	Valine	Vraisemblablement déplacement compétitif	croissance inhibée
ML 328 e	Leucine exigeant	Valine Isoleucine	Vraisemblablement déplacement compétitif	croissance inhibée
E 20 S, mutant valine-résistant	Résistant à la valine	Isoleucine	Déplacement compétitif	Rhl. Incorpore la valine exogène en lieu et place de l'endogène.

Table XV. — Effect of isoleucine and leucine on growth of mutant ML 328f exacting L-valine

L-valine M	L-isoleucine M	L-leucine M	Croissance (unités Nephelo)
I - 5.10^{-6}	-	-	14
10^{-5}	-	-	68
5.10^{-6}	10^{-6}	-	50
5.10^{-6}	$2,5.10^{-6}$	-	112
5.10^{-6}	5.10^{-6}	-	68
5.10^{-6}	10^{-5}	-	11
II - $2,5.10^{-6}$	-	-	27
5.10^{-6}	-	-	37
10^{-5}	-	-	57
$2,5.10^{-6}$	-	10^{-6}	38
5.10^{-6}	-	10^{-6}	45
5.10^{-6}	-	$2,5.10^{-6}$	57
5.10^{-6}	-	$2,5.10^{-5}$	19

Table XVI. -- Comparative sensibilities of growth of ML 328f on L-valine or on peptides containing L-valine and L-isoleucine.

L-valine M	DL-valyl-DL-phenylalanine M	DL-valyl-glycine M	DL-glycyl-valine M	L-prolyl-L-valine M	L-isoleucine M	DL-valyl-DL-valine M	L-valyl-L-valyl-valine M	Growth (Optical Density) τ
-	-	-	-	-	-	-	-	0
10^{-5}	-	-	-	-	-	-	-	114
10^{-5}	-	-	-	-	$2,5.10^{-4}$	-	-	0
-	10^{-5}	-	-	-	-	-	-	114
-	10^{-5}	-	-	-	10^{-2}	-	-	102
-	-	10^{-5}	-	-	-	-	-	107
-	-	10^{-5}	-	-	10^{-2}	-	-	95
-	-	-	10^{-5}	-	-	-	-	95
-	-	-	10^{-5}	-	10^{-2}	-	-	79
-	-	-	-	10^{-5}	-	-	-	79
-	-	-	-	10^{-5}	10^{-2}	-	-	64
-	-	-	-	-	-	10^{-5}	-	101
-	-	-	-	-	10^{-2}	10^{-5}	-	97
-	-	-	-	-	-	-	10^{-5}	65
-	-	-	-	-	10^{-2}	-	10^{-5}	66

The concentrations of peptides are given in M. When the peptides contain more amino acid optically active, the concentration is that of isomere L-L in making the hypothesis that the isomeres D-D, L-D, D-L are without action on growth. Essentially identical results are obtained with L-leucine as antagonist instead of L-isoleucine.